

Survival of *Gammarus troglophilus* (Gammaridae) after leg removal: evaluation of a procedure to obtain tissue for genetic analysis of rare and endangered amphipods

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Abstract. The removal of rare or endangered individuals from wild populations for evaluation of genetic composition may have deleterious consequences to these populations. As a viable alternative, we recommend limb removal as a means to obtain tissue for genetic analysis of rare amphipods. The effect of limb amputation on the survival of amphipods has not been previously studied. Our experiments showed that survival of *Gammarus troglophilus*, a common amphipod of Midwest caves and springs, was similar ($p=.74$) between control (unmanipulated) and experimental (amputation of 1 or 2 of the walking legs, pereopods 5 through 7) groups of amphipods. After 42 d, 25 of 26 amphipods in the experimental group had regenerated limbs that were half the size of the original appendage. Post-amputation survival could allow sampling of tissue for genetic analyses without sacrificing individuals, an important asset when working with species that are endangered or for which population sizes are very small.

Additional key words: Autotomy, autospasy, limb amputation, limb regeneration, endangered species

Potential genetic problems faced by small populations have been a central topic in conservation biology (Charlesworth & Charlesworth 1987) and researchers are expending considerable effort to determine the genetic composition of wild populations (e.g., Ciofi et al. 2002; Comstock et al. 2002; Waldick et al. 2002). Hair or fecal material from large animals provides sufficient tissue to allow genetic analysis (e.g., Paetkau et al. 1998; Palomares et al. 2002). However, for small-bodied invertebrates, the whole animal is usually processed. This presents a problem for rare or endangered invertebrates such as the Illinois cave amphipod *Gammarus acherondytes* HUBRICHT & MACKIN 1940, for which population sizes are unknown (U.S. Fish and Wildlife Service 2002) and protection of existing wild populations may be critical. Methods used to extract DNA from single rotifers (e.g., Leutbecher 2000), which are up to 3 orders of magnitude smaller (length and mass) than amphipods, could be used to obtain genetic information from amphipods or other small-bodied invertebrates by removing only a single ap-

pendage, provided the animal can survive such an amputation. Limb regeneration in response to autotomy, autotilly, and autospasy (Bliss 1960) is well known in Crustacea (McVean 1982) and has been well studied in large crustaceans, such as crabs and lobsters (McVean 1982), but we are unaware of any studies that specifically report on the survival of amphipods after the loss of a limb. Charniaux-Cotton (1957) investigated the regeneration of the second gnathopod in the beach hopper *Orchestia gammarella* Pallas 1766, but did not present survival data.

In shallow groundwater cave streams of the Salem Plateau karst region of southwestern Illinois, the endangered amphipod *G. acherondytes* co-occurs with *Gammarus troglophilus* Hubricht & Mackin 1940, which is similar in appearance. While the range of *G. acherondytes* is restricted to portions of Monroe and St. Clair counties, Illinois, *G. troglophilus* commonly occurs in caves and springs of counties bordering the Mississippi River in southwestern Illinois and eastern Missouri (Holsinger 1976). Survival of amphipods after leg removal could allow the acquisition of tissue samples to investigate population genetics of *G. acherondytes* without negatively influencing its abun-

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dance. Using *G. troglophilus* as a surrogate for the endangered *G. acherondytes*, we examined amphipod survival after leg amputation. We also quantified the degree of leg regeneration in animals surviving at the end of the experiment.

Methods

Collection site

Illinois Caverns (Monroe County) is located in the Salem Plateau karst region of southwestern Illinois, USA. Cave systems and underground streams are numerous in this region as indicated by the high density of sinkholes (>90 sinkholes/km²) (Panno et al. 2003). The surface drainage basin for Illinois Caverns is ~ 5.4 km² (Aley et al. 2000) while the cave itself contains ~ 9.6 km of passage, much of which contains a perennial cave stream. Surface land use in the drainage is primarily agricultural (row crops and livestock grazing), but recently includes urban housing developments. Discharge of water in the cave stream averages 4.88×10^{-3} m³·s⁻¹ (range 4.83×10^{-4} to 2.49×10^{-2} m³·s⁻¹) (Taylor et al. 2000). The stream substrate ranges from fine particles and clay in pools to large boulders in riffles. However, cobbles 3–5 cm in diameter predominate in areas where the stream channel is broad and the water is <10 cm deep. The mean annual water temperature is 13.2°C (range 11.8–15.6°C) (Taylor et al. 2000). Other physico-chemical parameters are summarized in Taylor et al. (2000).

Collections

Individuals of *Gammarus troglophilus* were collected from Illinois Caverns on 28 August 2002 with fine-mesh dip nets. Specimens were obtained primarily from pools in the cave stream starting ~ 50 m upstream of the main cave entrance where the substrate was composed of fine sediments with some medium (<50 cm diameter) rocks. Randomly selected individuals ($n=60$), spanning the most common size range present in the samples (range 8.3–22.2 mm total body length; immature and adults), were placed into 4-L containers filled with native stream water for transport to the laboratory, ~ 1.5 h distant. During transport, all jugs were placed in ice-packed coolers and aerated individually with an aquarium air stone supplied by a battery-powered pump. We also collected >200 L of additional water and enough small (3 to 5-cm diameter) rocks from the cave stream to supply holding and experimental aquaria with at least 2 rocks each. Dried leaves collected from the forest floor near the cave entrance served both as additional substrate and as food in holding and experimental aquaria. In the lab-

oratory, amphipods were transferred to 3, 8-L aquaria filled with water from the cave stream and allowed to acclimate for 48 h in complete darkness. All holding and experimental aquaria were housed in a walk-in environmental chamber maintained at $13.0 \pm 0.2^\circ\text{C}$.

Experimental protocol

To determine the survival of the amphipods after leg removal, we compared the number of amphipods surviving after 42 d between control groups (no manipulation) and experimental groups (leg removed). Experimental aquaria were set up immediately after we returned from collecting amphipods. Each of 6 rectangular (28 L \times 17 W \times 12 H cm) aquaria was filled with 3.9 L of 80 μm -filtered cave water, 2 rocks, and 1 leaf (Pin Oak *Quercus palustris* MÜNCHHAUSEN 1770). The rocks and leaf were gently shaken in filtered cave water to remove any attached macroinvertebrates before they were placed in the aquaria. After 48 h, 10 amphipods were transferred from the holding aquaria to each of the 6, 4-L aquaria. Care was taken to achieve an even distribution of sizes and gravid females among the aquaria. A random number system was then used to assign 3 aquaria each to either the control or experimental group and to determine their order of placement on the bench in the environmental chamber. Controls were not manipulated, whereas 1 or 2 legs were removed from each individual in the 3 experimental aquaria. To remove pereopods, we held amphipods in the mesh of a small aquarium net and used fine watchmakers forceps to grasp the basis of each pereopod. This ensured that all pereopods were removed at the same article (joint of coxa and basis). Our original intent was to remove only the 7th pereopod from each individual, but because amphipods moved continuously while out of water and pereopods were readily released by autotomy, 1 or 2 pereopods were removed on any of segments 5–7. All aquaria were checked once every 2–3 d until the end of the experiment on Day 42.

On each observation day, a diffuse indirect light was turned on near the aquaria for 3–15 min to count amphipods in each aquarium and add or change water and leaves. At all other times, the aquaria were maintained in complete darkness. Water that had evaporated was replaced with 80 μm -filtered cave water to maintain 3.9 L in each aquarium. On Days 10 and 24 of the experiment, 1 L of water was exchanged in each aquarium. In each aquarium, the leaf was removed and replaced with a whole leaf when the amphipods had skeletonized it. Leaves added to the aquaria had an average area of 98.7 cm² (± 8.4 cm² SE, standard error; $n=5$); a wet, dry, and ash-free dry mass of 1.97

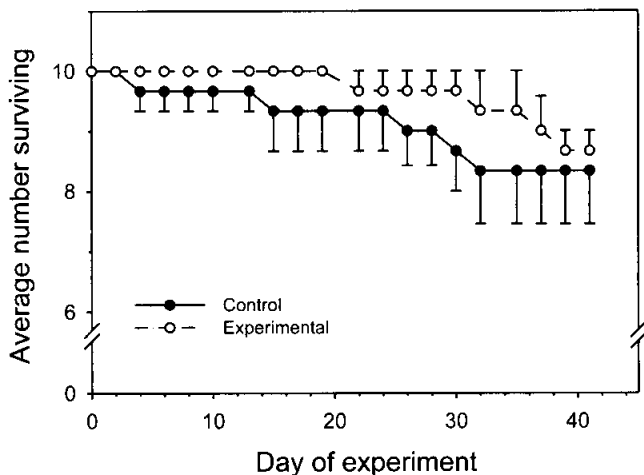


Fig. 1. Survival of the amphipod *Gammarus troglophilus* in control and treatment groups during the experiment. Individuals in replicates of the control group were not manipulated, while 1 to 2 pereopods were removed from each individual in replicates of the experimental group. Survival between treatments did not differ ($p=.74$, $t=.354$, $d.f.=3$). Error bars represent ± 1 SE; only half bars are presented for clarity.

g (± 0.13 SE), 0.75 g (± 0.08), and 0.69 g (± 0.07), respectively. Area was determined with a digitizer, while wet, dry, and ash-free dry mass were determined after briefly blotting leaves on paper toweling, drying to constant weight at 50°C, and ashing at 500°C for 3 h. Regardless of the degree of consumption, leaves were changed once on Day 10. After this, amphipods appeared to feed mainly on detritus that had accumulated on the bottom of the aquaria rather than on leaves. In aquaria in which <10 individuals were counted, leaves and rocks were gently removed to ensure individuals were not missed because they were hidden. Any dead or newly released individuals were removed from the aquaria. To determine if leg removal affected the survival of amphipods, we used a t -test to compare the number of amphipods remaining in each group at the end of the experiment.

To determine the size of amphipods and the degree of wound healing or leg regeneration, each individual remaining at the end of the experiment was photographed with a dissecting microscope (Leica MZ 9.5) equipped with a calibrated digital camera (Fuji FinePix 6800Z). Amphipods were then either returned to the collection site or used in other experiments. The total length of each individual was measured as the dorsal length from the tip of the rostrum to the base of the telson (Quigley & Lang 1989). To compare amphipod size among aquaria we used a 1-way analysis of variance (ANOVA). This allowed us to determine whether size influenced survival. Lengths were recorded after the experiment rather than before, to avoid

excessive handling of the amphipods and potentially biasing the experiment. To test if body size influenced regeneration, we performed a regression analysis of the percent regeneration as a function of body size.

The number and position of amputated legs were recorded. The length of regenerated legs was measured, with digital imaging software ImageJ (Rasband 2001), from the distal end of the basis to the distal end of the dactyl. This length was expressed as a percentage of the same distance on the original leg in the same position on the opposite side of the individual. Because we photographed live individuals, opposing legs on 4 individuals were not visible or blurred due to animal movement and could not be measured. Those animals were omitted from this analysis but their regrown legs were similar in size to those of other amphipods in the experimental group.

Average values of amphipod length and leg regeneration are reported as mean \pm SE. Voucher specimens have been deposited in the Southern Illinois University Invertebrate Collection and the Illinois Natural History Survey Crustacean Collection.

Results

Survival did not differ ($p=.74$, $t=.354$, $d.f.=3$) between treatments (Fig. 1). The average length of the amphipods was 18.7 mm \pm 0.6 and was similar ($p=.30$, $F_{5,44}=1.249$) between aquaria. Neither leg removal or animal length had a significant effect on survival.

Of the 9 deaths that occurred in the 2 groups (control, 5; experimental, 4), only 1 dead amphipod, a large male, was recovered. Field and laboratory observations suggest that the dead individuals probably were consumed by the remaining amphipods. During observations on and after Day 6, precopula pairs involving the largest individuals in an aquarium were noted in both groups. Newly released young were first seen and removed from aquaria starting on Day 22 after which the number of pairs declined. The presence of precopulatory pairs and then young suggests that female amphipods molted during the experiment. However, no exuvia were observed in aquaria of either group. Like the dead individuals, exuvia were probably consumed by the amphipods.

One amphipod in the experimental group did not regrow its leg and still had black scar tissue at the site of amputation on Day 42. Other individuals showed an average regeneration of the amputated limb of 50 \pm 2.2%, $n=21$ (Fig. 2) of the total length of the same pereopod on the opposite side of the animal. The particular limb removed (pereopod 5, 6, or 7) did not appear to influence the degree of regeneration. How-

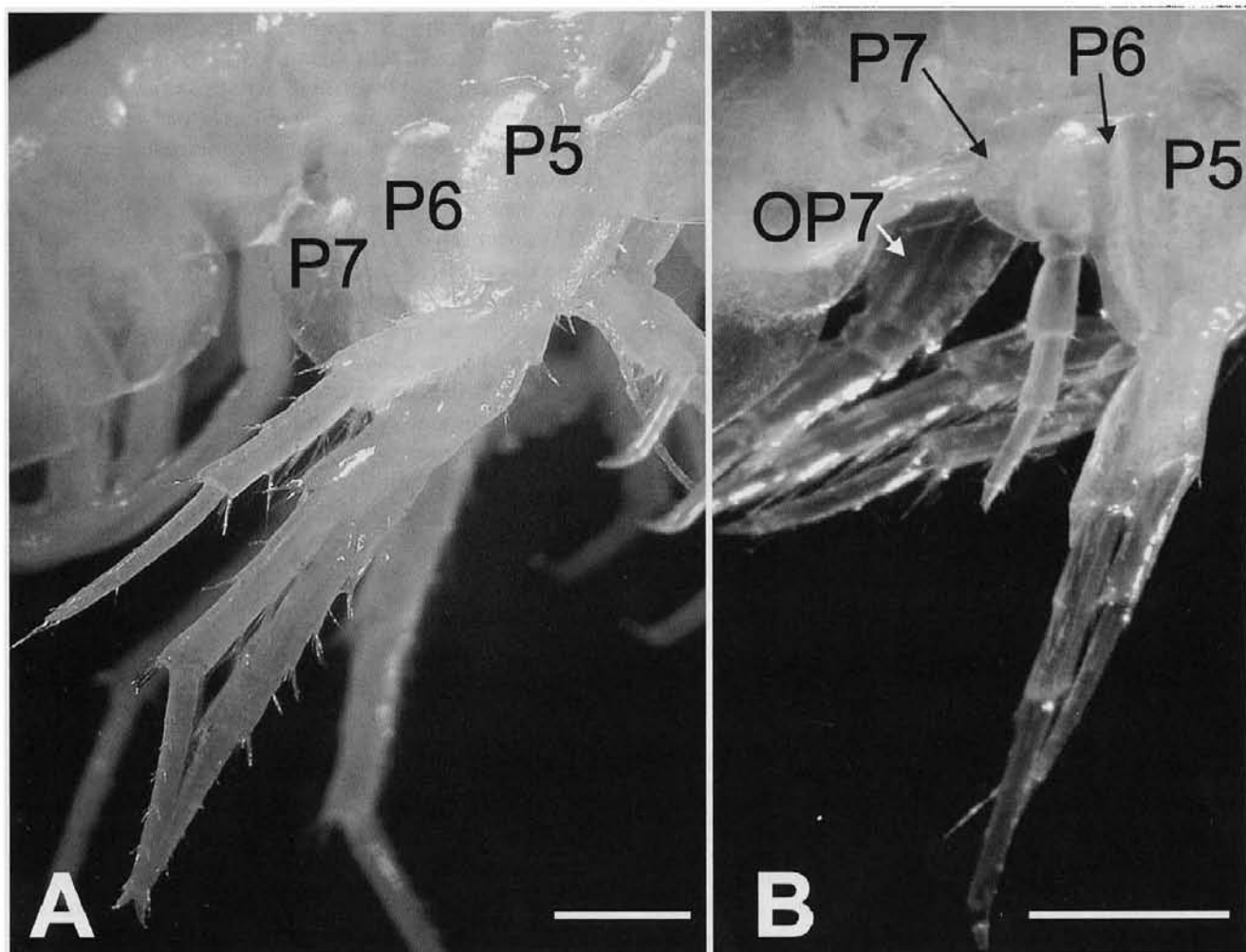


Fig. 2. Pereopods before amputation (A) and extent of regeneration after 42 d (B) in *Gammarus troglophilus*. P5–P7, pereopods 5 through 7; OP7—size of original pereopod on opposite side of the one amputated. Scale bars, 1 mm.

ever, 2 of the 3 amphipods that had 2 limbs removed showed the greatest amount of regeneration at 61 and 67%. There was no relationship between regeneration and body size; the slope of the relationship did not differ from zero ($p = .40$, $F_{1,19} = .740$). Removal of legs did not appear to negatively influence the activity patterns in *G. troglophilus* except that individuals on the bottom of the aquarium seemed to spend more time on their sides than upright compared to control animals.

Discussion

Our results show that specimens of *Gammarus troglophilus* can survive the removal of pereopods. The growth of new legs also indicates that, like other crustaceans, this species is capable of regenerating lost limbs (Skinner 1985; Hopkins 2001). Furthermore, pair formation and mating occurred after removal of

posterior pereopods, suggesting that amputation does not diminish mating success. Gnathopods 1 and 2 are primarily used by males to carry and guard females, before mating (Borowski 1984). Similarly, swimming and respiratory ventilation are functions of the pleopods, which are not affected by missing legs. Thus, loss of a posterior pereopod should not negatively impact amphipods.

The death of similar numbers of amphipods in both the control and the experimental groups suggests that leg removal was not the primary cause of death. Although the ecology of *G. troglophilus* in Illinois Caverns is not well known, their basic biology is probably similar to that of other gammarids. For example, large males of *Gammarus lacustris* (SARS 1863) generally die after mating (Wilhelm & Schindler 1996) and post-reproductive males are often quickly consumed by conspecifics (Wilhelm, unpubl. data). The deaths in our experiment probably represented senescent, post-

reproductive males because we noted fewer large non-gravid individuals in aquaria in which deaths occurred after Day 22. The 1 dead amphipod recovered was a male.

Although unlikely, survival was perhaps influenced by the laboratory environment and our experimental results do not accurately reflect survival under natural cave conditions. Cave environments are well known for long-term constancy in environmental parameters such as water temperature, ionic composition, and food—or lack thereof (Poulson & White 1969). The temperature throughout the experiment was 13.0°C, which was within 1°C of the temperature on the day amphipods were collected and within 0.2°C of the mean annual water temperature of the collection site (Taylor et al. 2000). In addition, we filled aquaria with native cave water to avoid complications due to ionic balance or the presence/absence of microflora or fauna. We did not replicate the stream flow due to logistic constraints. However, most of the amphipods were originally collected from deep pools with slow-moving water. Therefore, it is unlikely that the lack of the water flow in the aquaria influenced the amphipods to any great extent. We provided the amphipods with leaf material. Such material and other organic debris is regularly washed into the cave from surface runoff and is processed by the cave community. Given the similarity of our laboratory conditions to the natural cave environment, we feel confident that our results can serve as a reasonable simulation of field conditions.

The lack of a significant relationship between regeneration and body size was unexpected. Given that small individuals (juveniles) generally have a higher metabolic and growth rate (e.g., Peters 1983) and thus a shorter intermolt period compared to adults, we expected small individuals to display greater regeneration than large individuals. Perhaps all individuals in the size range we used in our experiment grew slowly, similar to other cave dwelling species (e.g., Culver et al. 1995).

The regeneration of new limbs to half the size of the original limb over the experimental period suggests that complete regeneration of the limbs could require ~80 d. Jenio (1980) found that the mean time from hatching to sexual maturity in culture was 210 d and calculated a life span of 360 d for spring-run populations of *G. troglophilus*. Ongoing experiments with individuals of *G. troglophilus* and observations on *G. acherondytes* from Illinois Caverns suggest they have a longer life span (Wilhelm, unpubl. data). Life cycles of other amphipods lasting 3 (*Gammarus lacustris*; Wilhelm and Schindler 1996) to 8–10 years (*Cranogonyx antennatus* Packard 1881; Holsinger & Holsinger 1971; Dickson & Holsinger 1981) suggest that a

life span greater than 1 year is not unrealistic for amphipods. Given these long life-cycles, it appears that regeneration of lost limbs may be a reasonable energy investment in *G. troglophilus*. The above-average regeneration by 2 of the 3 individuals that had 2 limbs removed is consistent with the general crustacean pattern of faster limb regeneration in cases of multiple vs. single limb loss (Skinner 1985; Hopkins 1993).

Given the similarities between *G. troglophilus* and *G. acherondytes*, there is no reason to expect that the response of *G. acherondytes* to the removal of pereopods would differ from that of *G. troglophilus*. In addition, the removal of legs 5, 6, or 7 did not appear to hinder mating or other activities. Therefore, we recommend the removal of these limbs as a strategy to collect tissue samples to assess the population genetics or for other molecular analyses of *G. acherondytes* and other small rare or endangered crustaceans.

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